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(54) Title: RECEPTOR FUNCTION ASSAYS

(57) Abstract

Methods are provided for assaying the function (e.g., activation) of signal-transducing receptors on cells. The methods are useful for identifying compounds that are agonists or antagonists of receptor function. The assays utilize a two-antibody sandwich assay employing an immobilized first antibody that specifically binds and captures the receptor of interest along with any tightly associated proteins or polypeptides and a second antibody directed against (i) an epitope of the receptor that is characteristic of the activated but not the unactivated state, (ii) an epitope of a protein or molecule characteristically bound to activated receptor but not to unactivated receptor, or (iii) an epitope of the receptor that is characteristic of the unactivated but not the activated state. In one embodiment, the level of binding of the second antibody is determined and correlated with the presence of activated receptor, and thus with activation in response to the treatment received by the cells. The methods provided by the invention will find particular use for detecting activation of tyrosine kinase growth factor receptors.

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RECEPTOR FUNCTION ASSAYS

BACKGROUND OF THE INVENTION

1. Field of the Invention

Signal-transducing receptors mediate fundamental aspects of cell physiology, growth, differentiation, endocrine, paracrine and exocrine signalling, and other properties of cells. Because of the importance of these receptors (and their cognate ligands) in human health, the identification of compounds that antagonize or agonize receptor activity will have profound consequences in medicine. The development of efficient, high capacity assays for such compounds is important for the development of therapeutic agents to treat human diseases.

Receptor-mediated signal transduction occurs when the receptor binds its ligand and is activated, initiating a variety of cellular responses. Although the particular responses to ligand binding depend on the receptor and cell type, the repertoire of early responses characteristic of receptor activation includes dimerization or oligomerization of multi-subunit receptors, receptor autophosphorylation, substrate phosphorylation, and receptor association with specific cellular proteins. As a consequence of receptor activation by ligand, subsequent changes in the cell physiology occur (involving, e.g., mitogenesis, specific gene activation, phosphatidylinositol turnover, phospholipase A2 activation, and changes in cell shape, cellular calcium concentration, and intracellular pH). In some cases, signal transduction and the resulting changes in cell physiology can be blocked by interference with an early response characteristic of receptor activation. For example, inhibition of the tyrosine kinase activity by phosphotyrosine-containing peptides can prevent signal transduction (Pazin & Williams, 1992). Similarly, when heterodimerization of wild-type fibroblast growth factor receptor (FGF-R) or platelet-derived growth factor receptor (PDGF-R) is prevented by expressing inactive, mutant receptors

subsequent signal transduction is blocked (Ueno et al., Science 252:844-848). It is also possible to specifically block certain receptor-mediated changes in cell physiology without affecting other aspects of signal transduction (See, e.g., Peters, K. G. et al. (1996) Nature 358:678-681; Escobedo, J.A. and Williams, L.T., (1988) Nature 335:85-87).

One class of signal-transducing receptors, the growth factor receptors, is of particular interest, due in part to the implication of some growth factor receptors in a wide range of human diseases (e.g., cancer, cardiovascular disease, and inflammatory diseases). Many growth factor receptors belong to the tyrosine kinase family of receptors, which includes platelet-derived growth factor receptors (PDGF-R), fibroblast growth factor receptors (FGF-R), and others.

Tyrosine-kinase activity of such growth factor receptors is induced by ligand binding to the receptor extracellular domain, and results in both autophosphorylation and substrate phosphorylation; additionally, transphosphorylation (the phosphorylation of one receptor subunit by another, associated, subunit) may occur. Activation of some growth factor receptors is also characterized by dimerization or oligomerization of receptor subunits. Activation is also characterized, for some receptors, by receptor association with specific cytoplasmic proteins.

Because of the fundamental role of signal transduction in biology and medicine, it would be very useful to have efficient methods for assaying receptor function. For example, an assay that measures traits characteristic of receptor activation (e.g., early responses such as receptor dimerization, receptor-protein association, and tyrosine kinase activity) would be desirable for identifying therapeutics targeted to receptors (e.g., growth factor receptors).

The methods presently available for measuring aspects of receptor function, such as dimerization and tyrosine phosphorylation, are suitable for an academic laboratory setting, but are not appropriate for high-throughput assays. For example, receptor dimerization has been measured by chemical cross-linking or immunoprecipitation followed by

Western blot analysis or autoradiography. Similarly, at present the primary means for identifying tyrosine phosphorylation of a specific protein within the cell is by immunoprecipitation of that protein followed by Western blot analysis using an antiphosphotyrosine antibody. These methods are difficult, expensive, and not suitable for screening large libraries of natural or synthetic compounds for activity. The availability of efficient, high throughput assays of receptor function will provide means to identify agonists and antagonists useful as therapeutic agents for the treatment of a wide variety of human diseases.

Description of the Background Art

Cleveland et al. (Analytical Biochem. 190:249, 1990) describe a enzyme-linked immunosorbent assay (ELISA) assay to measure phosphorylation by partially purified tyrosine kinases. This assay uses purified heterologous substrate protein or synthetic peptides immobilized on a microtiter plate. Others (e.g., Babcook et al. Analytical Biochem. 190:245, 1991; Ritjksen et al. Analytical Biochem. 182:98, 1989) have described similar assays for tyrosine kinase activity employing antiphosphotyrosine antibodies and synthetic peptide substrates.

Reed-Gitomer (U.S. Patent Application No. 07/341,949) describes an ELISA-based assay used to distinguish between various dimeric forms of platelet-derived growth factor (PDGF).

Lazarus et al. (Symp. Soc. Exp. Biol. (ENGLAND) 45: 129, 1991) describe a "sandwich" ELISA used to demonstrate that auxin binding induces a conformational change in an auxin-binding protein of maize. Keating et al. (J. Biol. Chem. 263:12805, 1988) used immunoprecipitation and gel electrophoresis to demonstrate phosphorylation-dependent conformational changes in activated PDGF receptors. Nelson et al. (J. Clin. Immuno. 6:114, 1986) used a sandwich ELISA for measuring soluble and cell-associated interleukin-2 receptors (IL2-R), to demonstrate increased IL2-R release and expression by stimulated lymphocytes.

SUMMARY OF THE INVENTION

The invention provides methods for assaying the function of signal-transducing receptors are that are useful for screening for agonists or antagonists of receptor activation. These methods utilize whole cells expressing the receptor of interest and comprise the steps of (i) treating the cells with agonists, antagonists, and/or test compounds, (ii) preparing a cell lysate from the treated cells, and (iii) assaying for receptor function using a two-antibody sandwich assay. The two-antibody sandwich assay employs an immobilized first antibody (ab1) that specifically binds and captures the receptor of interest along with any tightly associated proteins or polypeptides and a second antibody (ab2) directed against (i) an epitope of the receptor that is characteristic of the activated but not the unactivated state, (ii) an epitope of a protein or molecule characteristically bound to activated receptor but not to unactivated receptor, or (iii) an epitope of the receptor that is characteristic of the unactivated but not the activated state. An important characteristic of the first antibody (ab1) is that it captures (specifically binds) the receptor of interest whether activated or not activated by the treatment. The level of binding of the second antibody is determined and correlated with the presence of activated (or unactivated) receptor, and thus with activation (or lack of activation) in response to the treatment received by the cells. Thus, the quantity of receptors that are activated in cells can be determined. In some embodiments additional antibodies are used to quantitate the total amount of receptor present (allowing calculation of the fraction of total receptor in the activated state) and for detection of other antibodies. Thus, by determining the amount of activated receptor or the proportion of total receptor activated in variously treated cells, the agonizing or antagonizing activity of a compound can be determined. Similarly by determining the amount of unactivated receptor or the proportion of total receptor that is not in the activated state in variously treated cells, the agonizing or antagonizing activity of a

compound can be determined. These methods may be easily adapted to high-throughput formats (e.g., by using microtiter plates and colorimetric detection) making them ideally suited for the discovery of compounds that influence receptor activation.

The methods provided by the invention will find particular use for detecting activation of tyrosine kinase growth factor receptors as assessed by receptor and substrate phosphorylation, receptor dimerization and oligomerization, and receptor association with specific cytoplasmic proteins.

BRIEF DESCRIPTION OF THE DRAWINGS

Fig. 1 shows assay results showing induction of PDGF-R dimerization by PDGF.

Fig. 2 shows assay results showing induction of PDGF-R autophosphorylation by PDGF.

Fig. 3 shows assay results showing association of PDGF-R and phospholipase cγ in the presence of PDGF.

DESCRIPTION OF SPECIFIC EMBODIMENTS

Methods are provided for assaying receptor function. Generally, the receptors of interest will be growth factor receptors, but function of any receptor can be assayed by this method provided that the receptor has at least one epitope that is characteristic of the activated state but not the unactivated state or at least one epitope that is characteristic of the unactivated state but not the activated state, and at least one epitope that is characteristic of both the activated and unactivated (i.e., not activated) states. In a preferred embodiment the receptor will have at least two epitopes characteristic of both the activated and unactivated state.

For the sake of clarity, throughout the remainder of this disclosure, methods used to determine the amount of receptor in the activated state will be described (i.e., those methods employing an antibody directed against an epitope characteristic of the activated state but not the unactivated state). It will be apparent to one of ordinary skill in the art

that by appropriate substitution of an antibody directed against an epitope characteristic of the unactivated state but not the activated state, the amount of receptor in an unactivated state can be determined. The relationship between receptor in the activated and unactivated states is:

Total receptor= activated receptor + unactivated receptor.

A receptor is in an activated state when (i) it has bound a ligand (i.e., a natural ligand or other agonist that binds the receptor) and is transducing a signal (e.g., association with specific cytoplasmic proteins) in the manner characteristic of the binding of the natural ligand or (ii) the receptor is transducing a signal in the manner characteristic of the binding of the natural ligand even though no ligand is bound to the extracellular ligand binding site.

A tyrosine kinase growth factor receptor is in an activated state (a) if the tyrosine kinase activity of the receptor is elevated, (b) if receptor polypeptides are associated in dimers or oligomers, when such association is elevated when the receptor is bound by a natural ligand of the receptor, compared to unoccupied receptor, or, (c) if the receptor is tightly associated with the intracellular or membrane proteins whose association with the receptor is elevated when the receptor is bound to its natural ligand, compared to unoccupied receptor.

A ligand, as used herein, is a molecule or macromolecule that binds an extracellular region of a receptor. A "natural" ligand for a receptor is a ligand found in nature functionally associated with the receptor. For example, PDGF, in the forms found in nature, is a natural ligand of the PDGF receptors; insulin is a natural ligand of the insulin receptors, etc.

An epitope is the site on an antigen (e.g., a receptor) that is recognized by an antibody or antibody fragment. For the practice of this invention it is not necessary that the epitope bound be characterized in molecular terms. The epitope is characterized in functional terms (e.g., the association of the epitope with activated receptor).

An essential aspect of the invention is the use of cells that express the receptor of interest (hereafter referred to as "receptor-expressing cells"). The level of receptor expression will typically be at least 0.5×10^4 receptor polypeptides/cell, with at least 10^5 receptor polypeptides/cell more preferred, and at least 10^6 receptor polypeptides/cell most preferred. In a preferred embodiment, receptor-expressing cells are produced by transforming eukaryotic cells with a recombinant polynucleotide encoding the receptor polypeptide of interest (e.g., a receptor cDNA) to produce cells expressing or overexpressing the receptor polypeptides. In the most preferred embodiments, mammalian cells are used. Methods for recombinant expression of polypeptides are well known in the art (see, e.g., Sambrook et al. (1989) Molecular Cloning: A Laboratory Guide, Vols 1-3, Cold Spring Harbor Press, which is incorporated herein by reference). It will sometimes be desirable to express more than one polypeptide in a cell (e.g., for measuring dimerization of a heterodimeric receptor). In a second embodiment, a cell line that endogenously expresses a receptor can be identified by selection and screening. When selecting receptor-expressing cells, cells that express high levels of receptor will generally be preferred over cells expressing lower levels of receptor. Methods for assessing receptor expression levels are well known in the art and include immunological, binding competition, and functional assays (see, e.g., Methods in Enzymology, Vol. 198, 1991, Barnes, D., Mathers, J.P., Sato, G.H. (Eds.) Academic Press, Inc., which is incorporated herein by reference).

The assays of the instant invention are suited to a wide variety of receptors, but will be especially useful for assaying function of growth factor receptors, especially tyrosine kinase growth factor receptors. Examples of tyrosine kinase growth factor receptors include platelet-derived growth factor receptors (PDGF-R), fibroblast growth factor receptors (FGF-R), insulin receptor (In-R), epidermal growth factor receptor (EGF-R), erythropoietin receptor (EPO-R), colony stimulating factor receptor (CSF-R), stem cell factor receptor (SCF-R), vascular endothelial growth factor receptor (VEGF-R),

FLK-1-receptor, FLT-1-receptor, ERB-2-receptor and ERB-3-receptor.

According to the method of the assay, receptor-expressing cells will be grown under conditions appropriate for growth and proliferation and for expression of the receptor of interest. General tissue culture techniques are well known in the art and are described in, e.g., Methods in Enzymology, Vol. 58, 1979, Jakoby, W. B. and Paston, I. (Eds.), Academic Press, Inc., which is incorporated herein by reference. Media appropriate for eukaryotic cells are well known and readily available. Typically, the cell medium will be supplemented with serum (e.g., fetal bovine serum, FBS), which provides growth factors and essential nutrients. However, in some cases, complete media that are not supplemented with FBS will be preferred because all the components are known and such media can be obtained free of contaminating growth factors and/or compounds that interact with the receptor of interest and which may affect the assay. In a preferred embodiment, cells will be grown in multiwell cell culture plates (sometimes referred to as microtiter plates) suitable for cell cultures, because of the ease of screening large numbers of samples using these plates. Usually 96-well cell culture plates will be used (e.g., Falcon 96-well cell culture plates; Costar 96-well cell culture plates, cat. # 3596). Cells will be seeded according to usual tissue culture practices at an appropriate cell density. This density will depend on cell-type, but typically will be about 10^4 cells/well for adherent cells in 96-well microtiter plates. If possible, cells should be seeded so that they reach confluency in 1-2 days.

The cells will be grown to about confluency. For cells grown in the presence of serum, it will sometimes be desirable to incubate the confluent cells for about 24 hours (at least 6 hours and typically not more than 48 hours) in a serum-free medium in order to remove growth factors and/or compounds that interact with the receptor of interest. However, often this will not be necessary or desirable because the medium in which cells are incubated is typically depleted of growth factors during the process of cell growth and

proliferation. Usual tissue culture practices are followed in exchange of medium and other aspects of cell culture. For example, in shifting from serum-containing to serum-free media, cells will typically be "washed" with a physiological buffer or with serum-free medium in order to effectively remove residual serum.

The assay can also be carried out using nonadherent cells, by making modifications that will be apparent to one of ordinary skill in the art. For example, non-adherent cells can be grown and incubated with test compounds in suspension, and can be centrifuged and resuspended according to usual practice to exchange media, collect for cell lysis, etc (see, e.g., Methods in Enzymology Vol. 58, supra).

To assay for compounds that act as receptor function agonists or antagonists, the cells are exposed to known agonists, known antagonists, and/or test compounds which may be, or may contain, agonists or antagonists. An agonist, antagonist, or test compound may be a chemical compound, a mixture of chemical compounds, a biological macromolecule, or an extract made from biological materials such as bacteria, plants, fungi, or animal cells or tissues. Test compounds are evaluated for potential activity as agonists or antagonists of receptor activation by inclusion in screening assays described herein. An "agonist" enhances the activity of a receptor; an "antagonist" diminishes the activity of a receptor. The terms "agonist" and "antagonist", as used herein, do not imply a particular mechanism of function.

According to the method of the assay, when assaying for agonist activity, receptor-expressing cells will be incubated with a known agonist, a test compound, or a combination of the two. In a preferred embodiment, the cells are incubated with the agonist or test compound for about 1 hour at about 4 °C. However, the time and temperature of incubation may range from about 1 minute to about 5 hours, and from about 4 °C to about 37 °C, but will usually be at least enough time for receptor activation by the natural ligand, if such time known. Lower temperatures (e.g., 4 °C) are generally preferred to reduce the possibility of proteolysis,

dephosphorylation, dissociation of associated proteins or other processes that could interfere with the assay. When higher temperatures (e.g., 37°C) are used, a shorter incubation time will usually be desirable (e.g., 10 minutes) to minimize these interfering processes. During the incubation of cells with agonists and/or test compounds, the receptor will be activated if there is a suitable receptor agonist present (e.g., in the test compound) and no antagonist present.

When assaying for antagonist activity, receptor-expressing cells will be incubated with a known antagonist, a test compound, or a combination of the two, and, in addition, will be incubated with an agonist. According to the assay, the presence of the agonist will cause receptor activation unless antagonist is present.

The order of addition of antagonist (or test compound) and agonist can vary. The agonist and antagonist may be added to the receptor-expressing cells simultaneously, or, alternatively, the antagonist (or test compound) can be added before addition of the agonist

The conditions (time and temperature) for incubations when testing for antagonist activity are essentially the same as those described to agonists, supra. However, when they are added in sequence total time will not usually be longer than five hours.

It may also be possible to use receptor-expressing cells that express a receptor variant that is in a constitutively activated state (i.e., activated in the absence of ligand; see, e.g., Roussel et al. (1990) Oncogene 5:25-30). In this case, an assay for a compound with receptor antagonist activity will not necessarily require incubation with a known agonist.

The amount or concentration of agonist/antagonist added will, when known, vary depending on the compound, but will generally range from about 10pM to 100μM. Typically, a variety of concentrations will be used. In the case of uncharacterized test compounds it may not be possible, and it is not necessary, to determine the concentration of agonist/antagonist.

It will also be desirable to include various experimental controls in the assay. Examples of appropriate controls include negative controls and positive controls. In testing for agonist activity, negative controls can include incubation of cells with inert compounds (i.e., compounds known not to have agonist activity) or in the absence of added compounds. Positive controls can include incubation of cells with compounds known to have agonist activity (e.g., the natural ligand). Logically similar (though complementary) controls can be included in assays for antagonist activity, as will be apparent to one of ordinary skill in the art of biology, as will be various additional controls. The description of controls is meant to be illustrative and in no way limiting.

After exposure of the cells to test compounds, a cell lysate is prepared. Methods for preparation of a cell lysate are well known in the art (see, e.g., Methods in Enzymology Vol. 198, supra). Typically, cells are homogenized in the presence of buffers (e.g., Tris-HCl, HEPES), nonionic detergents (e.g., Tween-20; Triton X-100), and protease inhibitors (e.g., PMSF, aprotinin, leupeptin). Other inhibitors, such as phosphatase inhibitors (e.g., orthovanadate), may also be desirable. Typically conditions are chosen that are nondenaturing to the cellular proteins of interest. A preferred method for preparation of a cell lysate is by addition of lysis buffer (20mM Tris-HCl, pH 7.3, 150 mM NaCl, 1% Triton X-100, 1mM PMSF, 1mM sodium orthovanadate, 10 μg/ml aprotinin and 10 μg/ml leupeptin) followed by cell disruption (e.g., by shaking or scraping). Usually, when preparing a cell lysate the insoluble matter in the cell lysate is removed by centrifugation (e.g., 10,000 xg for 15 min.) and the clarified supernatant is recovered.

In a preferred embodiment, the resulting cell lysate is transferred to a well of a microtiter plate to which an antibody (ab1) against the receptor of interest is immobilized. Methods for immobilizing antibodies are known in the art and are described in Harlow, E. and D. Lane, Antibodies: A Laboratory Manual, (1988), Cold Spring Harbor Laboratory, Cold

Spring Harbor, New York, (hereafter referred to as "Harlow"), which is incorporated herein by reference. The antibody may be either polyclonal or monoclonal. The antibody (ab1) will be selected as able to specifically bind ("capture") the receptor in both activated and unactivated states. Typically the antibody will bind the receptor with an affinity of at least about 10^{-7} M^{-1}.

In an alternative embodiment, a cell membrane fraction, especially a plasma membrane fraction can be purified from the cells treated with a test compound or, e.g., a known agonist or antagonist, using standard methods (see, e.g., Methods in Enzymology Vol. 198, supra.) and used in the assay. One advantage of using a membrane preparation will be that the purified membranes can be frozen and stored for a period of time, which may, in some cases, increase the convenience of the assay and allow use of a cell membrane preparation to be used for several assays over a period of time. When using a membrane fraction in the assay it will sometimes be useful to further disrupt the membrane fraction before adding combining the membranes and antibodies.

Antibodies, either monoclonal or polyclonal, can be generated to receptor polypeptides or peptides in a variety of ways known to those skilled in the art including injection of antigen into animals, by hybridoma fusion, and by recombinant methods involving bacteria or phage systems (see, e.g., Marks et al. (1992) J. Biol. Chem. 267:16007; Marks et al. (1992) Biotechnology 10:779; Lowman et al. (1991) Biochem. 30:10832; Lerner et al. (1992) Science 258:1313; each of which is herein incorporated by reference).

Antibodies may be produced by immunizing an appropriate vertebrate host, e.g., mouse, with the antigen (e.g., a receptor polypeptide, peptide, or extracellular domain), itself or in conjunction with an adjuvant. Usually two or more immunizations will be involved, and the blood or spleen will be harvested a few days after the last injection. For polyclonal antisera, the immunoglobulins may be precipitated, isolated and purified, including affinity purification. For monoclonal antibodies, the splenocytes

normally will be fused with an immortalized lymphocyte, e.g., a myeloid line, under selective conditions for hybridomas. The hybridomas may then be cloned under limiting dilution conditions and their supernatants screened for antibodies having the desired specificity.

A variety of screening strategies are suitable including antibody capture, antigen capture and functional assays (see, e.g., Harlow). One preferred method for screening monoclonals and polyclonal antibodies that bind receptor is antibody capture. Techniques for producing antibodies are well known in the literature and are exemplified by U.S. Patent Nos. 4,381,292, 4,451,570, and 4,618,577, and are described in Harlow, supra. Typically, an antibody directed against a receptor will have a binding affinity of at least $1x10^7$ M^{-1}.

In a preferred embodiment, the first antibody (ab1) is directed against the extracellular domain of the receptor of interest; this is advantageous because the possibility that the epitope bound by the first antibody (ab1) would interfere with the binding of a candidate second antibody (ab2) directed to an intracellular epitope of the receptor (e.g., a phosphotyrosine) or a cytoplasmic region of receptor association with other proteins is reduced.

The cell lysate and the first antibody (ab1) will be incubated together for a time and at a temperature sufficient to allow binding of the receptor and antibody. Typically, this will be about 30 minutes to about 12 hours at a temperature of 4°C to 37°C.

Following this incubation, the unbound material will be removed by gently washing the wells with a suitable buffer such as one containing 25 mM HEPES pH 7.6, 100 mM NaCl, and 0.2% Tween 20. For general descriptions of methods used in two-antibody sandwich assay methods see, e.g., Harlow, Chapter 14; Ling and Overby, 1972, J. Immunol. 109:834-841; Belanger et al., 1973, Clin. Chim. Acta 48:15-18; Mailolini and Masseyeff, 1975, J. Immunol. Methods 8:223-234; each of which is herein incorporated by reference.

A second antibody (ab2) that is specific for an epitope characteristic of the activated form of the receptor,

or for an epitope of a protein that is tightly associated with the activated, but not unactivated receptor, is added to the well and allowed to bind under suitable conditions for antibody binding, as described for ab1-receptor binding, supra. As noted, supra, epitopes characteristic of an activated receptor can include sites of tyrosine phosphorylation. Thus, in some embodiments, an antibody against phosphotyrosine would be a suitable second antibody (ab2). Such tyrosine phosphorylation can be on the receptor polypeptide bound by the first antibody (ab1; e.g., autophosphorylation) or on a tightly associated protein. The identity of tightly associated proteins will vary according to the receptor being studied but can include a second receptor polypeptide (e.g., in homodimers and heterodimers and oligomers) or other proteins (e.g., phosphoinositide-3-kinase, GTPase activating protein, phospholipase Cγ [PLCγ] and nonreceptor tyrosine kinases such as those of the src family [e.g., $pp60^{c\text{-}src}$, $p59^{fyn}$, and $pp62^{c\text{-}yes}$]). A protein is considered to be tightly associated with a receptor if the receptor and protein remain associated in the presence of a nonionic detergent (e.g., Tween-20 nonionic detergent, Triton X-100 nonionic detergent). Usually a tightly associated protein remains associated with a receptor in the presence of a nonionic detergent at a concentration of 0.1% (v/v), more usually at a concentration of about 0.25%, still more usually about 0.5%, even more usually at about 0.75%, and most usually at a concentration of about 1% (v/v). Such tight association will typically be determined under conditions of near physiological salt (e.g., 0.1 - 0.2 M NaCl) and pH (e.g., 7.2 - 7.6). Typically, any polypeptide or biomolecule that is associated with the activated, but not unactivated receptor, and which remains associated with the receptor when captured by the first antibody (ab1) will be useful in the assay of the invention and will be considered tightly associated.

In other embodiments, the second antibody (ab2) can be directed against other protein modifications characteristic of an activated receptor (e.g., phosphorylation, cleavage). In further embodiments, the second antibody (ab2) can be directed against an epitope of a protein or other biomolecule (e.g., a

lipid, polysaccharide, glycoprotein, glycolipid, peptide, polypeptide, nucleic acids and other biological macromolecules) tightly associated with an activated receptor.

Following the incubation with the second antibody (ab2), the unbound material will be removed by gently washing the wells. In a preferred embodiment, the second antibody (ab2) will be labeled so that bound antibody can be detected and, if desired, quantitated. A number of methods for detecting antibodies are known in the art (see, e.g., Harlow, supra) including labeling with an enzyme (e.g., alkaline phosphatase, horseradish peroxidase β-galactosidase) with a fluorochromes, with radioactive markers, with biotin, and by other methods. Depending on the label chosen, various means known in the art can be used to detect and/or binding by the second antibody (ab2) (see, e.g., Harlow, supra). In a most preferred embodiment, the second antibody (ab2) is conjugated with peroxidase which is detected, e.g., by monitoring product formation at 650 nm following the addition of ABTS (2,2'-azinobis (3-ethyl-benzthiazoline-6-sulfonic acid).

An alternative method for detection of the second antibody (ab2) involves using a third antibody (ab3) directed against the second antibody (ab2). According to this embodiment, the second antibody (ab2) is not itself labeled but is dictated by binding of the third antibody (ab3). The third antibody (ab3) can be labeled as described for the second antibody (ab2), supra. This alternative method for detection will be particularly preferred when the first (ab1) and second (ab2) antibodies have different xenotypes (e.g., mouse and goat), and may allow increased sensitivity of the assay.

By detecting the second antibody (ab2), either directly or indirectly, the quantity of activated receptors can be determined. "Quantity of activated receptors", as used herein, can refer to a measurement of the actual number of activated receptors per cell (or the equivalent) or to a qualitative comparison between two (or among several) measurements. For example, in making a qualitative comparison it will be possible to determine that there are more activated

receptors in one well than there are in a second well, without measuring the actual number of receptors in either well.

In a preferred embodiment of the invention, a fourth antibody (ab4) is used in the assay to quantitate the total amount of receptor captured. The fourth antibody (ab4) is directed against an epitope characteristic of both activated and unactivated receptor. Furthermore, the fourth antibody must be able to bind receptor that is bound (i.e., captured) by the first antibody (ab1) and bound by the second antibody (ab2). Thus, although the specificity of the first (ab1) and fourth (ab4) antibodies is similar, if used in combination they must be able to bind the receptor polypeptide at the same time.

According to the assay, by comparing the binding of the second antibody (ab2) and the fourth antibody (ab4) an index of activation can be determined. The index of activation (IA) is defined as follows:

IA = activated receptor bound/total receptor bound = moles ab2 bound/ moles ab4 bound

In a preferred embodiment of the invention, receptor activation of a tyrosine kinase growth factor receptor is measured. In one variation of this embodiment, the second antibody (ab2) is directed against phosphotyrosine.

In a second variation of this embodiment, the first antibody (ab1) recognizes a first monomer of a homo- or hetero-dimer or oligomer, and the second antibody (ab2) recognizes a second monomer of a homo- or hetero- dimer or oligomer, and the first and second monomers are tightly associated only or primarily when the receptor is in an activated state (i.e., when such association is elevated when the receptor is bound by its natural ligands, compared to unoccupied receptor).

In a third variation of this embodiment, the second antibody is directed against a protein (other than a second receptor subunit or polypeptide) that is tightly associated with the receptor only or primarily when the receptor is in an activated state.

It will be apparent to one of ordinary skill in the art that variations of the aforedescribed embodiments are feasible. In less preferred embodiments, for example, the first antibody (ab1) will be immobilized on solid phase substrates other than a microtiter plate (e.g., cell culture beads). In another embodiment, the order of addition of the first (ab1) and second (ab2) antibodies will be reversed. According to this embodiment, a second antibody (ab2) is added to a cell lysate prepared as described above, and allowed to bind an epitope characteristic of activated receptor (e.g., phosphotyrosine). The lysate-antibody mixture including ab2-phosphorylated protein complex, if present, is then combined with the immobilized first antibody (ab1) which captures the receptor along with any second antibody (ab2) molecules bound to the receptor or to a molecule tightly associated with the receptor (e.g., a second monomer of a receptor dimer). The second antibody (ab2) is then quantitated as a measure of activated receptor.

The following experimental section is offered by way of example and not by limitation.

EXAMPLES

The following Examples describe the application of the assays of the invention using cells expressing platelet-derived growth factor (PDGF) receptor(s).

I. Development of antibodies

Monoclonal antibody against the PDGF receptor (anti-β-PDGF-R 1C7D5) was prepared according to standard methods (see, e.g., Harlow) using recombinant extracellular domain protein as previously described (Ramakrishnan et al. (1993) Growth Factors 8:253-265). Polyclonal antibodies against PDGF α (anti-α-PDGF-R-3980) and β (anti-β-PDGF-R-3982) receptor extracellular domains were made in rabbits using recombinant extracellular domain protein (Fretto et al., supra) according to standard procedures (see, e.g., Harlow, supra). These antibodies were shown not to crossreact with beta PDGF receptor. Anti-phosphotyrosine antibodies were obtained from

commercial sources (International Biotechnologies, Inc.). Anti phospholipase C-γ-1 was obtained from Upstate Biotechnology Inc. (UBI). Conjugated antibodies directed against mouse monoclonal antibodies or rabbit polyclonal antibodies used for colorimetric detection were obtained commercially. Horseradish peroxidase (HRP)-conjugated anti-rabbit IgG (Boehriger Mannheim) and HRP-conjugated monoclonal antibody PY20 (International Biotechnologies, Inc.) were obtained commercially.

II. Identification of cell lines for assay development

A. Non-recombinant cell lines

To identify a cell line expressing high levels of each PDGF receptor subtype, a collection of human tumor cell lines obtained from the American Type Culture Collection were screened for receptor levels. The cell lines screened included (ATCC numbers in parentheses): SKLMS1 (HTB88), A204 (HTBA2), HS68 (CRL1635), MG63 (CRL1427), HS27 (CRL1634), WS1 (CRL1502), HT1080 (CRL121) and G402 (CRL1440). PDGF receptor levels were measured using a two-antibody sandwich assay (see, Harlow, supra). Briefly, 1 μg of monoclonal antibody specific for either alpha or beta PDGR receptor (see above) was immobilized in wells of microtiter dishes. Cell lysates for the tumor lines were prepared essentially as described in Example III, infra, and were incubated in the wells to allow for capture of PDGF receptor. The wells were washed, and rabbit polyclonal antibodies directed against the PDGF receptor were added and allowed to bind to captured receptor. The level of polyclonal antibody bound was detected enzymatically using horseradish peroxidase conjugated species anti-rabbit antibody source. The sarcoma cell line, MG 63, was shown to express high levels of both alpha and beta PDGF receptor.

B. Recombinant Cells

To develop a sensitive assay to measure beta PDGF receptor phosphorylation, CHO cells overexpressing recombinant beta PDGF receptor were used (HR5 cells, Escobedo et al. (1988)

Science 240:1532-1534, which is incorporated herein by reference).

III. Assays to Measure Receptor Function

A. Receptor Dimerization Assay

To measure the dimerization between alpha and beta PDGF receptors, MG 63 cells were seeded at 300,000 cells/well in 6-well dishes (Falcon tissue culture plates) and incubated at 37°C in 2 ml/well of Dulbecco's minimum essential media (DMEM) with 10% fetal bovine serum for 3 days at which time cells were confluent. The media was removed from the wells and replaced with serum-free DMEM and incubation was continued at 37°C for 18 hours. To induce PDGF receptor dimerization, PDGF AA, AB or BB was added at 2-200 ng/ml to each well containing 700 µl DMEM and cells were incubated for 2 hours at 4°C. The media was drained and 60 µl of freshly prepared lysis buffer (20 mM Tris at pH 7.3, 150 mM NaCl, 1% Triton X-100, 1 mM PMSF, 1 mM sodium orthovanadate, 10 µg/ml aprotinin and 10 µg/ml leupeptin) was added to each well and cells were scraped from the wells to prepare the cell lysate.

In a separate microtiter plate, monoclonal antibody 1C7D5, which reacts specifically with beta PDGF receptor was immobilized by incubating 0.7 µg of antibody per well at 4°C for >18 hours (5 days) in 23 mM Tris at pH 8.0, 68 mM NaCl, 14 mM ammonium bicarbonate and .01% sodium azide. After antibody immobilization, the wells were blocked for 2 hours at room temperature with 25 mM HEPES pH 7.6, 100 mM NaCl, and 0.2% Tween 20 just prior to the addition of cell lysate. Cell lysate was incubated with this immobilized antibody for 160 minutes at 4° to allow antibody capture of beta PDGF receptor polypeptides and tightly-associated proteins, and the wells were washed 3 times with 150 µl of wash buffer (0.3% gelatin, 25 mM HEPES, 100 mM NaCl and 0.01% Tween 20 at pH 7.4).

If alpha PDGF receptor is in a complex with beta PDGF receptor following ligand stimulation, both receptor subtypes will be captured by the immobilized antibody. To detect alpha receptor that is captured in a complex with beta receptor, polyclonal anti-alpha-PDGF-R (3980) was added to the washed

wells (0.29 μg/well in 100 μl of wash buffer) and incubated for 105 minutes at 4°C. After washing the wells 3 times with 150 μl of wash buffer, 100 μl of horseradish peroxidase conjugated anti-rabbit IgG (Boehringer Mannheim cat. # 1238850) was added to each well at a 1 to 1000 dilution. The plates were incubated for 1 hour at 4°C and wells were washed 3 times with 150 μl of wash buffer and once with 200 μl phosphate buffered saline. 100 μl/well of peroxidase substrate (ABTS, supra) was added and product formation was monitored spectrophotometrically at 650 nM using a microtiter plate reader (Molecular Devices). When PDGF AA is added, no alpha PDGF receptor was detected in complex with beta receptor (Fig. 1). These results are consistent with previous studies using chemical crosslinking that conclude that alpha/beta PDGF receptor dimers do not form in the absence of ligand or in response to PDGF AA stimulation (Kanakara et al. Biochem. 30:1761, 1991). In contrast, stimulation of MG 63 cells with PDGF AB and BB produced a dose-dependent increase in the detection of alpha PDGF receptor indicating that alpha/beta PDGF receptor complexes had formed (Fig. 1), which is consistent with previous reports using chemical crosslinking (Kanakara et al., supra). PDGF AB and BB responses were observed at concentrations as low as 0.17 nM and were maximal at 4 nM. This is the concentration range at which PDGF mitogenic responses occur in vivo, demonstrating that the assay is functioning at physiologically relevant concentrations.

B. Receptor Phosphorylation Assay

To measure the beta PDGF receptor phosphorylation, HR5 cells (see above) were seeded at 10,000 cells/well in microtiter plates (Falcon 96 well plates) and incubated at 37°C in RPMI with 10% fetal bovine serum for 3 days at which time confluency was reached. The media was removed from the wells and replaced with 100 μl of serum free RPMI and incubation was continued at 37°C for 18 hours. To induce PDGF receptor phosphorylation, 0.05-2.0 ng of PDGF AA or BB (Fretto et al. (1993) J. Biol. Chem 268:3625-363) was added to each well containing 100 μl RPMI and the cells are incubated for 90

minutes at 90 minutes at 4°C. The media was drained and 50 µl of freshly prepared lysis buffer (20 mM Tris at pH 7.3, 150 mM NaCl, 1% Triton X-100, 1 mM PMSF, 1 mM sodium orthovanadate, 10 µg/ml aprotinin and 10 µg/ml leupeptin) was added to each well and the plate was shaken vigorously to prepare the cell lysate.

In a separate microtiter plate, monoclonal antibody 1C7D5 directed against the beta PDGF receptor was immobilized by incubating 1.0 µg of antibody per well at 4°C for 18 hours in 23 mM Tris at pH 8.0, 68 mM NaCl, 14 mM ammonium bicarbonate and .01% sodium azide. After antibody immobilization, the wells were blocked with 25 mM HEPES pH 7.6, 100 mM NaCl, and 0.2% Tween 20 just prior to the addition of cell lysate. The cell lysate was incubated with immobilized antibody against beta PDGF receptor for 2-3 hours at 4°C and wells are washed 3 times with 200 µl of wash buffer (0.3% gelatin, 25 mM HEPES, 100 mM NaCl and .01% Tween 20 at pH 7.4). To detect the level of beta PDGF receptor phosphorylation, horseradish peroxidase conjugated monoclonal antibody PY20 (International Biotechnologies, Inc.) directed against phosphotyrosine was added at 0.12 µg/well in 100 wash buffer and incubated for 1 hour at 37°C. The wells were drained and washed 3 times with 200 µl of wash buffer, peroxidase substrate (ABTS) in 100 µl of phosphate buffered saline was added and product formation is monitored as described, supra. In the presence of PDGF BB, beta PDGF-R phosphorylation is detected (Fig. 2) When no ligand or PDGF AA is added, no beta PDGF receptor phosphorylation is detected. These results are consistent with previous studies demonstrating that in the absence of PDGF, beta PDGF receptor contains little phosphotyrosine, and that PDGF AA does not activate this receptor (Kanakara et al., supra). In contrast, stimulation of HR5 cells with PDGF BB produced a dose dependent increase in the detection of beta PDGF receptor phosphorylation. PDGF BB responses were observed at concentrations as low as 0.2 nM and were maximal at 2-3 nM. This is the concentration range at which PDGF mitogenic responses occur, demonstrations that this assay is functioning physiologically relevant concentrations.

C. Assay to Measure Association of Receptor with Effector Molecules

To develop an assay to measure the binding of the beta PDGF receptor to phospholipase C-gamma (PLC gamma), HR5 cells were seeded at 5 million cells per 100 mm tissue culture dish (Falcon) in RPMI 1640 containing 10% fetal bovine serum and grown at 37°C for 3 days at which time the cells had reached confluency. The media was replaced with serum-free RPMI 1640 and incubation was continued for 18 hours at 37°C. The media was removed and 3 ml of RPMI 1640 or RPMI 1640 containing PDGF BB (50 ng/ml) was added and the incubation continued for 10 minutes at 37°C. The media was drained and 250 ul of freshly prepared lysis buffer (20 mM Tris at pH 7.3, 150 mM NaCl, 1% Triton X-100, 1 mM PMSF, 1 mM sodium orthovanadate, 100 μg/ml aprotinin and 10 μg/ml leupeptin) was added to each 100 mm plate and cells were scraped from the plates and collected into a microfuge tube. The microfuge tube was vortexed and centrifuged in a microfuge for 10 minutes at 4°C to remove insoluble material.

In a microtiter plate (Immulon II, Dynatech cat. # 011-010-3455), rabbit polyclonal IgG directed against the beta PDGF receptor (see above) was immobilized by incubating 0.5 ug of antibody per well in 100 ul of 23 mM Tris at pH 8.0, 68 mM NaCl, 14 mM ammonium bicarbonate and .01% sodium azide at 4°C for 18 hours. After antibody immobilization, the wells were washed once with 150 ul of 25 mM HEPES pH 7.6, 100 mM NaCl, and 0.2% Tween 20 and then 200 μl/well of this buffer was added and incubated at room temperature for 1 hour to block the wells. The wells were washed 2 times with 150 μl/well of incubation buffer (0.3% gelatin, 25 mM HEPES, 100 mM NaCl and 0.01% Tween 20 at pH 7.6) and 50 μl of cell lysate plus 50 μl of incubation buffer were added to each well and the plates were incubated for 2.5 hours at room temperature. The plates were washed 3 times with 150 ul/well of incubation buffer and 0.05 ug of a mixed preparation of mouse monoclonal antibodies directed against PLC gamma (Upstate Biotechnology, Inc.) was added to each well in 100 μl of incubation buffer and the plates were incubated at 37°C for

1 hour. The wells were washed 3 times with 150 µl of incubation buffer and 100 µl/well of a 1:1000 dilution of horseradish peroxidase coupled anti-mouse antibody (Sigma) was added and incubated at 37°C for 1 hour. The wells were washed 3 times with 150 µl of incubation buffer and 1 time with 150 µl of phosphate buffered saline. 100 µl/well of peroxidase substrate (ABTS, supra) was added and product formation was monitored at 650 nM (Molecular Devices).

When cell lysates were analyzed under these conditions, lysates prepared from HR5 cells that were stimulated with PDGF gave a 3-fold increase in signal as compared to lysates from cells that had not been stimulated with PDGF (Fig. 3). These results demonstrate that the association of PLC gamma with activated beta PDGF receptor occurring under physiological conditions can be subsequently assayed in a microtiter format that makes possible high throughput screening of synthetic or natural product libraries such that compounds that enhance or inhibit receptor/substrate interactions may be identified.

All publications and other references or patent documents herein are incorporated by reference. It is to be understood that the above description is intended to be illustrative and not restrictive. Many embodiments will be apparent to those of skill in the art upon reviewing the above description. The scope of the invention should, therefore, be determined not with reference to the above description, but should instead be determined with reference to the appended claims, along with the full scope of equivalents to which such claims are entitled.

WHAT IS CLAIMED IS:

1. A method for determining the ability of a test compound to affect function of a tyrosine kinase receptor by acting as an antagonist or an agonist of receptor activation, comprising the steps:

i) obtaining tyrosine kinase receptor-expressing cells;

ii) incubating a first portion of the tyrosine kinase receptor-expressing cells with a test compound to produce test cells and incubating a second portion of the tyrosine kinase-expressing cells in the absence of the test compound to produce control cells;

iii) determining the quantity of receptors that are activated in the test cells and in the control cells by using a two-antibody sandwich assay comprising the steps:

a) lysing the cells;

b) capturing the receptor with an immobilized first antibody (ab1) that binds the receptor; and

c) determining the quantity of activated receptors by binding a second antibody (ab2) that recognizes phosphotyrosine, wherein phosphotyrosine is characteristic of the activated tyrosine kinase receptor;

iv) comparing the quantity of activated receptors in the test cells with the quantity of activated receptors in control cells; and

v) correlating a difference in quantity of activated receptors in the test cells with the quantity of activated receptors in control cells with the ability of the test compound to affect function of the receptor, wherein an increased quantity of activated receptors in the test cells indicates receptor agonist activity by the test compound and a decreased quantity of activated receptors in the test cells indicates receptor antagonist activity by the test compound.

2. A method for determining the ability of a test compound to affect function of a receptor by acting as an antagonist or an agonist of receptor activation, comprising the steps:

i) obtaining receptor-expressing cells;

ii) incubating a first portion of the receptor-expressing cells with a test compound to produce test cells and incubating a second portion of the receptor-expressing cells in the absence of a test compound to produce control cells;

iii) determining the quantity of activated receptors in the test cells and in the control cells by using a two-antibody sandwich assay comprising the steps:

a) lysing the cells;

b) capturing the receptor with an immobilized first antibody (ab1) that binds the receptor; and

c) determining the quantity of activated receptors by binding a second antibody (ab2) that recognizes a biomolecule tightly associated with the activated receptor but not tightly associated with unactivated receptor;

iv) comparing the quantity of activated receptors in the test cells with the quantity of activated receptors in control cells; and

v) correlating a difference in quantity of activated receptors in the test cells with the quantity of activated receptors in control cells with the ability of the test compound to affect function of the receptor, wherein an increased quantity of activated receptors in the test cells indicates receptor agonist activity by the test compound and a decreased quantity of activated receptors in the test cells indicates receptor antagonist activity by the test compound.

3. The method of claim 2, wherein the biomolecule is a polypeptide.

4. The method of claim 3, wherein the polypeptide is a receptor polypeptide.

5. The method of claim 3, wherein the polypeptide is phosphoinositide-3-kinase, GTPase activating protein, or phospholipase Cγ.

6. The method of claim 1, comprising the additional step of incubation of the tyrosine kinase receptor-expressing cells with a receptor-activation agonist at the same time as, or following, the incubation of step ii.

7. The method of claim 2, comprising the additional step of incubation of the receptor-expressing cells with a receptor-activation agonist at the same time as, or following, the incubation of step ii.

8. The method of claim 1, 2, 6, or 7, wherein the receptor-expressing cells express a growth factor receptor.

9. The method of claim 8, wherein the growth factor receptor is a platelet-derived growth factor receptor.

10. The method of claim 1 or 2, wherein the first antibody (ab1) is immobilized on a microtiter plate.

11. A method for determining the ability of a test compound to affect function of a receptor by acting as an antagonist or an agonist of receptor activation, comprising the steps:

i) obtaining receptor-expressing cells;

ii) incubating a first portion of the receptor-expressing cells with a test compound to produce test cells and incubating a second portion of the receptor-expressing cells in the absence of the test compound to produce control cells;

iii) determining the quantity of receptors that are activated in the test cells and in the control cells by using a two-antibody sandwich assay comprising the steps:

a) lysing the cells to form a cell lysate;

b) combining the cell lysate and a second antibody (ab2) that recognizes phosphotyrosine, wherein phosphotyrosine is characteristic of the activated receptor, or that recognizes a biomolecule tightly associated with the activated-receptor but not tightly associated with unactivated-receptor, wherein an activated-receptor-second antibody (ab2) complex is formed;

c) capturing the activated-receptor-second antibody (ab2) complex with an immobilized first antibody (ab1) that binds the receptor;

iv) comparing the quantity of activated receptors in the test cells with the quantity of activated receptors in control cells; and

v) correlating a difference in quantity of activated receptors in the test cells with the quantity of activated receptors in control cells with the ability of the test compound to affect function of the receptor, wherein an increased quantity of activated receptors in the test cells indicates receptor agonist activity by the test compound and a decreased quantity of activated receptors in the test cells indicates receptor antagonist activity by the test compound.

12. A method for determining the ability of a test compound to act as an antagonist or an agonist of receptor activation, comprising the steps:

i) obtaining receptor-expressing cells;

ii) incubating a first portion of the receptor-expressing cells with a test compound to produce test cells and incubating a second portion of the receptor-expressing cells in the absence of the test compound to produce control cells;

iii) determining the quantity of activated receptors that are activated in the test cells and in the control cells by using a two-antibody sandwich assay comprising the steps:

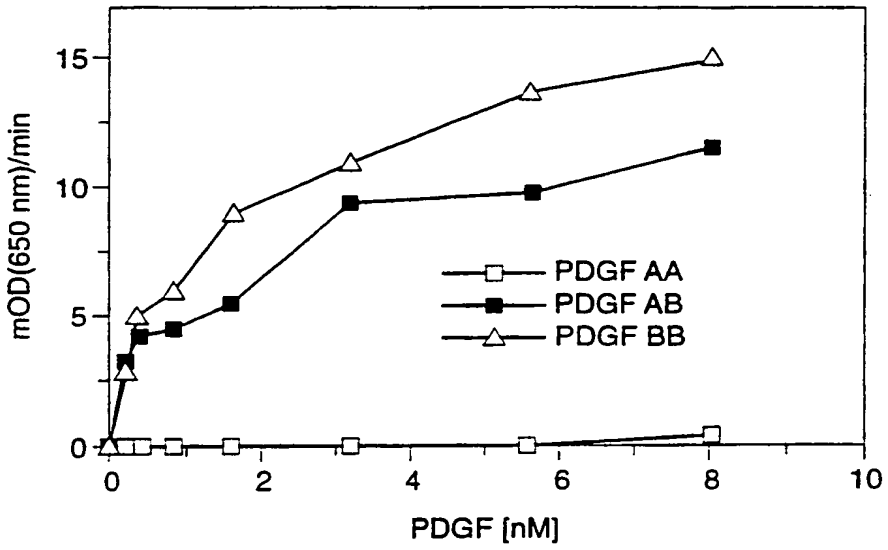
a) lysing the cells;

b) capturing the receptor with an immobilized first antibody (ab1) that binds the receptor; and

c) determining the quantity of activated receptors by binding a second antibody (ab2) that recognizes an epitope characteristic of receptor activation wherein the epitope characteristic of receptor activation is due to a covalent modification to the receptor or cleavage of the receptor;

iv) comparing the quantity of activated receptors in the test cells with the quantity of activated receptors in control cells; and

v) correlating a difference in quantity of activated receptors in the test cells with the quantity of activated receptors in control cells with the ability of the test compound to affect function of the receptor, wherein an increased quantity of activated receptors in the test cells indicates receptor agonist activity by the test compound and a decreased quantity of activated receptors in the test cells indicates receptor antagonist activity by the test compound.


mOD(650 nm)/min
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10
5
0
0
2
4
6
8
10
PDGF [nM]
PDGF AA
PDGF AB
PDGF BB

FIG. 1

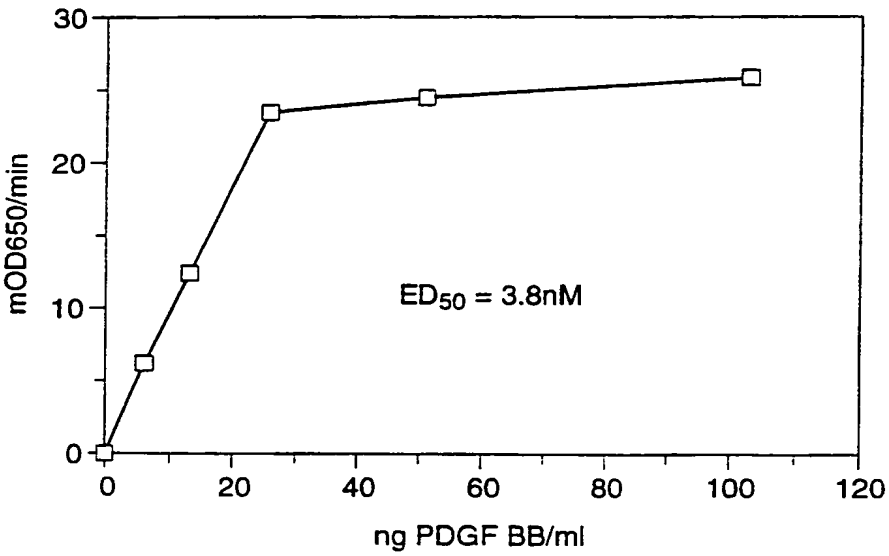
mOD650/min
30
20
10
0
0
20
40
60
80
100
120
ng PDGF BB/ml
ED_{50} = 3.8nM

FIG. 2

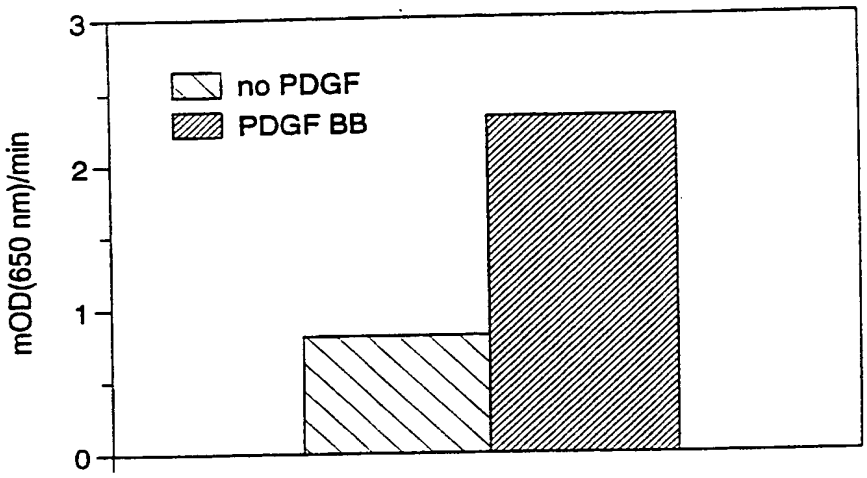

FIG. 3

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US94/08571

A. CLASSIFICATION OF SUBJECT MATTER

IPC(5) :C12N 9/12; G01N 33/53, 33/543, 33/545, 33/573
US CL :435/7.21, 7.4, 7.94, 194; 436/518, 531
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/6, 7.1, 7.21, 7.23, 7.24, 7.4, 7.94, 194; 436/518, 531; 514/885

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, DIALOG

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	CELL, Volume 58, issued 25 August 1989, D.K. Morrison et al, "Direct Activation of the Serine/Threonine Kinase Activity of Raf-1 through Tyrosine Phosphorylation by the PDGF β-Receptor", pages 649-657, see especially Figures 1B and 5 and page 656.	1-12
Y	CELL, Volume 69, issued 26 June 1992, N.Y. Ip et al, "CNTF and LIF Act on Neuronal Cells via Shared Signaling Pathways That Involve the IL-6 Signal Transducing Receptor Component gp130", pages 1121-1132, see especially Figures 5, 6D, 7A and pages 1124-1125 and 1130.	1, 6, 8, 10-12

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E"	earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search	Date of mailing of the international search report
23 OCTOBER 1994	03 NOV 1994
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231	Authorized officer JAMES L. GRUN, PH.D.
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INTERNATIONAL SEARCH REPORT

International application No.
PCT/US94/08571

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	SCIENCE, Volume 247, issued 30 March 1990, A. Kazlauskas et al, "Binding of GAP to Activated PDGF Receptors", pages 1578-1581, see especially Figure 1 and page 1579.	2-5, 7-12
Y	CELL GROWTH AND DIFFERENTIATION, Volume 2, issued December 1991, P.A. Thompson et al, "The Raf-1 Serine-Threonine Kinase Is A Substrate for the $p56^{lck}$ Protein Tyrosine Kinase in Human T-Cells", pages 609-617, see especially Figures 1C and 1E and page 616.	2-4, 8, 10-12
Y	US, A, 5,045,455 (KUO ET AL) 03 SEPTEMBER 1991, see entire document, especially columns 4 and 11-13.	1-12
Y	US, A, 4,376,110 (DAVID ET AL) 08 MARCH 1983, see entire document especially columns 1-2, 4, and 6.	10-11
Y	E. HARLOW et al, "ANTIBODIES, A LABORATORY MANUAL", published 1988 by COLD SPRING HARBOR LABORATORY (Cold Spring Harbor), page 605, see entire document.	10-11
Y	SCIENCE, Volume 252, issued 03 May 1991, C.A. Koch et al, "SH2 and SH3 Domains: Elements That Control Interactions of Cytoplasmic Signaling Proteins", pages 668-674, see especially pages 668-671.	5